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Studies on the microflora of millipedes (Diplopoda).
I. A comparison of actinomycetes isolated from surface structures of the exoskeleton and the digestive tract

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With one figure

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1. Introduction

Millipedes are vegetarian and feed on dead and decaying leaves. Being predominantly woodland organisms they mostly occur in the surface region of soils, especially in the litter layer, where they can play a decisive role not only in the plant litter decomposition, but also in microbial dissemination and in mixing and inoculating soil microhabitats with bacteria and fungi which they carry on their body surfaces. Although their feeding habits and digestion have been studied in detail by many authors (Nicholson et al., 1966; Marcuzzi 1970; Wooten & Crawford 1975; Pobozsny 1978; Kheirallah 1979; etc), we have, at present, only very few data on population composition at the specific level, properties of their intestinal bacterial populations and on their role in the passive dispersal of microbes. In the next issues of this journal we intend to present results from studies of the bacterial inhabitants, ecto- and endocommensals and microsymbionts of these animals. In the present study an attempt is made to compare the actinomycetes living, as stable members of the gut community, in dense populations in the digestive canal of millipedes, with those detected by light- or electron microscopy on various surface structures of the exoskeleton of Diplopoda.

2. Materials and methods

2.1. Animals involved in comparative investigations

Specimens of *Leptoiulus proximus* Nemec, *Chromatoiulus projectus* Verh., *Cylindroiulus luridus* (C. L. Koch), *Cylindroiulus boleti* (C. L. Koch) and *Unciger foetidus* (C. L. Koch) were collected (August, 1977 and July, 1980) from the litter layer of brown forest soils (Wald-Braunerde) in the Vértes mountains, about 40 km west of Budapest, at an elevation of approximately 170 m above sea level.

2.2. Isolation of actinomycetes from faecal material

Serial dilutions prepared from gut contents or fresh excrement samples obtained aseptically under laboratory conditions were plated onto glucose-ammonium phosphate medium [glucose 10..0 g $(NH_4)_2HPO_4$ 4.0 g, NaCl 5.0 g, K_2HPO_4 2.0 g, $MgSO_4$ 1.0 g, $CaCl_2$ 0.4 g, $FeSO_4 \cdot 7\,H_2O$ 0.02 g, $MnSO_4 \cdot 7\,H_2O$ 0.01 g, agar 20.0 g, distilled water 1000 ml, *p*H 7.2]; nutrient agar [peptone 5.0 g, beef extract 3.0 g, agar 20.0 g, distilled water 1 000 ml, *p*H 7.2); starch-casein medium [soluble starch 10.0 g, casein 1.0 g, Na_2HPO_4 0.5 g, agar 15.0 g, distilled water 1000 ml, *p*H 7.5]; and the glycerol-arginine medium of El-Ñakeeb & Lechevalier (1963) [arginine-monohydrochloride 1.0 g, glycerol 12.5 g, K_2HPO_4 1.0 g, NaCl 1.0 g, $MgSO_4 \cdot 7\,H_2O$ 0.5 g, $Fe_2(SO_4)_3 \cdot 6\,H_2O$ 0.010 g, $CuSO_4 \cdot 5\,H_2O$ 0.001 g, $ZnSO_4 \cdot 7\,H_2O$ 0.001 g, $MnSO_4 \cdot H_2O$ 0.001 g, agar 15.0 g, distilled water 1000 ml, *p*H 6.9—7.1]. Isolations were made randomly, and the isolates were transferred onto slopes of oatmeal agar. After purification, the cultures were maintained on oatmeal and/or on starch-casein agar. The plate counts of bacteria and actinomycetes on each medium were also estimated.

2.3. Isolation of actinomycetes adhering to surface structures of the legs

Electron microscopy showed that the very intricate surface structures of the millipede legs provide innumerable microsites available for colonization, or much more for passive adherence of soil microbes. The direct examinations of freshly collected millipede specimens by light microscopy have revealed, that on the legs, especially between the spine-like processes of the tarsal region, there are always large numbers of actinomycete conidia and bacterial cells more or less loosely dispersed. To isolate these surface colonizers and passive passengers, a method was used which approximated to the process by which the millipedes inoculate soil particles with the bacteria, which adhere to the surface of their metachronously moving legs. The individual animals were repeatedly placed into the surface of glucose-ammonium phosphate, nutrient, starch-casein and glycerol-arginine agar plates. These media were inoculated by the legs of the millipedes in decreasing density with the length of the period on the agar surface. After aseptically removing the animal, the plates were incubated at 28 °C for 3—12 days. Isolation, purification and maintenance of isolated organisms were carried out as outlined under 2.2.

2.4. Comparison of the isolated strains

The isolated and purified actinomycete strains proved to be the members of different genera. Therefore appropriate methods were used to distinguish them.

For the identification of *Streptomyces* strains the methods and media used in the International Streptomyces Project (SHIRLING & GOTTLIEB, 1966; SZABÓ et al. 1975) were used. For the identification of gut-isolates, micromorphological properties in wet-chamber agarfilm-cultures and cultural features on agar plates were studied. In addition to the ISP-media, Czapek's sucrose agar, yeast-extract starch agar [yeast-extract 10.0 g, soluble starch 20.0 g, agar 15.0 g, distilled water 1000 ml, *p*H 7.0] proposed by LUEDEMAN (1971) for the maintenance of *Micromonospora* strains, beef-extract-peptone agar [beef-extract 0.3 g, peptone 1.0 g, agar 20.0 g, distilled water 1000 ml, *p*H 7.0] and simple water-agar [agar 20.0 g, tap-water 1000 ml] were also used. Decomposition of cellulose was tested in the presence of yeast-extract [yeast-extract 5.0 g, $CaCO_3$ 1.0 g, agar 15.0, distilled water 1000 ml, *p*H 7.0] and without it in synthetic media of different composition with cellulose [0.5 %, MACHEREY & NAGEL, MN 300] as sole source of carbon. Starch hydrolysis was determined using a medium containing peptone 5.0 g, beef-extract 5.0 g, NaCl 5.0 g, starch 10 g, agar 15.0 g, distilled water 1000 ml, *p*H 7.0. Nitrate reduction was tested in nitrate broth [yeast-extract 5.0 g, glucose 10.0 g, KNO_3 1.0 g, $CaCO_3$ 1.0 g, distilled water 1000 ml]. Proteolytic potentialities were tested in gelatin-glucose medium and in skim milk agar. The potentialities to utilize various carbon compounds was determined partly in yeast-extract agar (LUEDEMAN 1971), and partly in Pridham and Gottlieb's synthetic basal medium (SHIRLING & GOTTLIEB 1966). Temperature range of growth was observed on yeast-extract-starch agar. Sensitivity to antibiotics was determined by the use of sensitivity disks manufactured by the Institute for Serobacteriological Production and Research "Human", Budapest.

2.5. Methods used for detecting the presence of gut actinomycetes in the litter layer

At the sampling sites of millipedes, litter-samples rich in decaying and partly decomposed plant leaves were also collected. These underwent the same bacteriological quantification and isolation procedures as used for detecting the composition of the faecal actinomycete community.

3. Results and discussion

A comparison of the relative frequency of actinomycetes within the total bacterial communities of the freshly collected faecal material, the consumed litter matter and on surface of the legs, was made for *Chromatoiulus projectus*, using starch-casein agar for plating and isolations. This resulted in 18, 2.3 and 95 % frequencies, respectively. These data were obtained in the course of our first study with millipedes, in 1977. Later (in 1980) we found frequencies of actinomycetes up to 34 % in the faecal matter of *Ch. projectus*. The litter layer in which millipedes live always contains actinomycetes which are predominantly streptomycetes. Also the wet or dry leaves consum~~p~~ed by the animals are loosely covered with streptomycete hyphae and conidia. It is interesting that the relative frequency of actinomycetes is highest on the body surface of millipedes. In the gut they are still quite frequent, constituting a part of the enormously increased gut bacterial community. The litter, and within this, the decaying plant materials produced relatively low counts of actinomycetes. The qualitative analyses of the actinomycete populations of the above-mentioned three habitats showed that on the surface structures of the legs and other appendices of the exoskeleton. *Streptomyces* species (presumably in the form of conidia) are the predominant organisms. By contrast, in the faecal flora they are only sporadically occurring aliens. It is not yet clear in which part of the millipede digestive canal streptomycetes are killed, but it is clear that their numbers decrease markedly during the gut passage. Also their numbers are extremely low or even zero in the freshly collected faecal pellets. Consequently, in the population dynamics of streptomycetes in the millipede milieu there are two contrasting processes, their accumulation on the body surface and their elimination from the intestinal canal.

The data in Table 1. clearly show that the *Streptomyces* strains identified from among the isolates of the agar surface prints of *Chromatoiulus projectus* legs represent a wide range of species. Only very few true bacteria were isolated from these plates. Therefore we conclude that the surface structures of these animals tend to select mostly for streptomycete conidia, rather than vegetative bacterial cells.

Ruddick & Williams showed (1972) that attraction occurs between the strongly hydrophobic spores of *Streptomyces griseus* and the hydrophobic cuticle of arthropods. Spores of *S. griseus* are dispersed very efficiently, carried externally rather than in the gut contents, by soil arthropods.

In the intestinal canal of millipedes there is a significant increase in the total number of actinomycetes and in their relative frequency within the gut bacterial community. In the case of *Ch. projectus*, this relative increase in frequency was considerable. It ranged from 2.3% in the consumed litter matter to 18% in the hind gut and fresh faecal pellets, from our first observations in 1977. These intestinal actinomycetes do not belong to Streptomycetaceae. They constitute, in the gut of the individual animal specimens, taxonomically very homogeneous populations, in the case of *Ch. projectus*, they constitute one species. These gut actinomycetes show close morphological similarity to the otherwise very rarely occurring genus *Promicromonospora* Krassilnikov et al. 1961. Analysis of the gut flora of specimens of five different millipede species collected in 1980, showed a similar situation. In the fresh faecal pellets, the ratio of bacteria to actinomycetes was relatively narrow and the actinomycetes consisted predominantly (except of a few sporadically occurring unidentified types and true streptomycetes) of organisms which were in many respects identical with those first isolated from the faeces of *Ch. projectus* (Table 2). These actinomycetes, designated as *Promicromonospora*, seem to be true inhabitants of the millipede intestinal canal, as they were not isolated from the animals' feeding habitats, nor did they occur on the body surface of Diplopoda.

On the basis of a detailed study of more than 50 strains of these *Promicromonospora*, type gut inhabitants isolated from the faeces and gut of *Ch. projectus* in 1977, we can characterize them as follows. All strains produce strongly developed yellow or yellowish substrate mycelium. The sessile spores occur singly on the individual hyphae of about 1 μm diameter (Fig. 1). They do not produce aerial mycelium. In the presence of peptone or yeast-extract the substrate hyphae do not remain intact, but quickly fragment into coccoid or longer elements. In such cases the yellow colonies are glistening, soft, pasty and seemingly very similar to true bacteria. Nitrates are reduced to nitrites, but they do not show proteolytic activities and cellulose decomposition. They can grow at *p*H 3.0, in 5% NaCl and at 30 °C. With inorganic N-compounds as sole nitrogen sources in the media, they utilize many simple carbon compounds such as fructose, glucose, mannose, lactose, sucrose, dextrin, etc. All of them are resistant to colistin, nalidic-acid, nystatin, etc., and sensitive to carbenicillin, spiramycin and polymyxin-B. Although the individual strains show some differences in carbon utilization, resistance to antibiotics, urease and phosphatase activities [illegible] that they constitute varieties of a single variable species. This species is capable of occupying a dominant position in the microbiota of the digestive canal of *Ch. projectus*. The bacteria-like appearance of these strains on peptone containing media, their complete fragmentation into coccoidal cells and their potency for fermentative breakdown of glucose under anaerobic conditions are distinguishing features among actinomycetes.

Table 1. Description of selected *Streptomyces* spp. isolated from agar-surface prints of moving

Strain No.	Specific epithet	Sporophore morphology	Spore surface morphology as seen in electrone microscope	Colour series of aerial mycelium (Tresner-Backus system)	Colour series of substrate mycelium (Szabó-Marton system)	Soluble pigment
D-ny-A-16	St. albogriseolus group	S	Sm	Gray	Y-b	None
D-ny-A- 8	St. flavidovirens ?	RA	Sm	White	Y-b	Yellowish
D-ny-A-12	St. flavidovirens	RA	Sm	White	Y-b	None
D-ny-A-10	St. fulvoviolaceus	RF	Sm	Gray	Y-b + red	Yellowish
D-ny-A-15	St. galtieri	RF	Sm	White	Y-b	Yellowish
D-ny-A-11	St. melanosporofaciens	RA	Sm	Gray	Y-b	None
D-ny-A- 7	St. nitrosporeus	RF	Sm	Gray	Y-b	None
D-ny-A-17	St. nitrosporeus	RF	Sm	Gray	Y-b	None
D-ny-A-19	St. nitrosporeus	RF	Sm	Gray	Y-b	Yellowish
D-ny-A-32	St. nitrosporeus	RF	Sm	Gray	Y-b	Yellowish
D-ny-A-33	St. nitrosporeus	RF	Sm	Gray	Y-b	Yellowish
D-ny-A- 4	St. nojirensis	S	Sm	Gray	Y-b	None
D-ny-A- 3	St. parvus group	RF	Sm	Yellow	Y-b	Yellowish
D-ny-A- 9	St. plicatus	S	Sm	Red	Y-b	Yellowish
D-ny-A-22	St. rubiginosohelvolus	RF	Sm	Red	Y-b	None
D-ny-A-13	St. spadicis	RF	Sm	Gray	Y-b	None
D-ny-A-23	St. umbrosus	S	Sm	Gray	Y-b	Yellowish
D-ny-A-24	St. umbrosus	S	Sm	Gray	Y-b	Yellowish
D-ny-A- 2	Streptomyces sp. I.	RF	Sm	Red	Y-b	Yellowish
D-ny-A-18	Streptomyces sp. II.	RF	Sm	?	Y-b + red	None
D-ny-A-26	Streptomyces sp. III.	RF	Sm	Gray	Y-b	Yellowish
D-ny-A-30	Streptomyces sp. IV.	RF	Sm	Gray	Y-b + red	Yellowish
D-ny-A-31	Streptomyces sp. V.	RF	Sm	Gray	Y-b + red	Yellowish

Abbreviations: St. = Streptomyces; RF = Rectiflexibiles; S = Spiral; RA = Retinaculi aperti

Table 2. The ratio of bacteria to actinomycetes in the total bacterial flora and the frequency of occurrence of *Promicromonospora*-type organisms within the actinomycete fraction in the fresh faecal material of different species of millipedes*

Animal Species	Bacteria: Actinomycetes	Promicromonospora-type organisms in the actinomycete fraction (%)
Leptoiulus proximus	3:1	>97
Chromatoiulus projectus	2:1	>94
Cylindroiulus luridus	3:1	>98
Cylindroiulus boleti	1:1	>86
Unciger foetidus	3:1	>94

*) Plate count technique was used. The cells of individual colonies on the agar plates were examined morphologically before counting. For the identification and further studies of Promicromonospora type organisms occurring in the feaces of all species of millipedes in these series of investigations (in 1980) altogether 250 strains were isolated. These are maintained in our culture collection. The plating media were: glucose-ammonium phosphate-, nutrient-, starch-casein- and glycerol-arginine-agar.

specimens of *Chromatoiulus projectus* Verh.

Melanoid pigment		C-source utilization								
Pepton-iron agar	Tyrosine agar	D-glucose	sucrose	D-xylose	i-inositol	D-mannitol	D-fructose	rhamnose	raffinose	L-arabinose
—	—	—	—	—	+	+	—	—	—	+
—	—	—	—	—	—	—	—	+	+	+
—	—	+	—	+	—	—	—	+	+	+
+	+	+	—	+	+	+	—	+	+	+
—	—	+	—	—	—	—	—	—	—	—
—	—	+	—	+	+	+	—	+	+	+
—	—	+	—	+	—	—	—	+	+	+
—	—	+	—	—	—	—	—	+	—	+
—	—	+	—	+	—	—	+	+	+	+
—	—	—	—	—	—	—	—	+	—	+
—	—	+	—	—	—	—	—	+	—	+
+	+	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	+	+	+	—	+
—	—	+	—	+	+	+	+	+	—	+
—	—	—	—	+	—	—	+	+	—	+
+	—	+	—	—	+	—	—	+	+	+
+	+	+	—	+	—	+	—	+	—	+
—	+	+	—	—	—	+	—	+	—	+
—	+	+	—	—	—	+	—	+	—	+
—	—	+	—	—	+	—	—	—	—	—
—	+	+	—	+	—	—	—	+	+	+
—	—	+	—	—	—	—	—	—	—	—
+	—	+	—	—	—	—	—	—	—	—

Y-b = Yellow-brown; Sm = Smooth; + = Positive utilization; — = No growth.

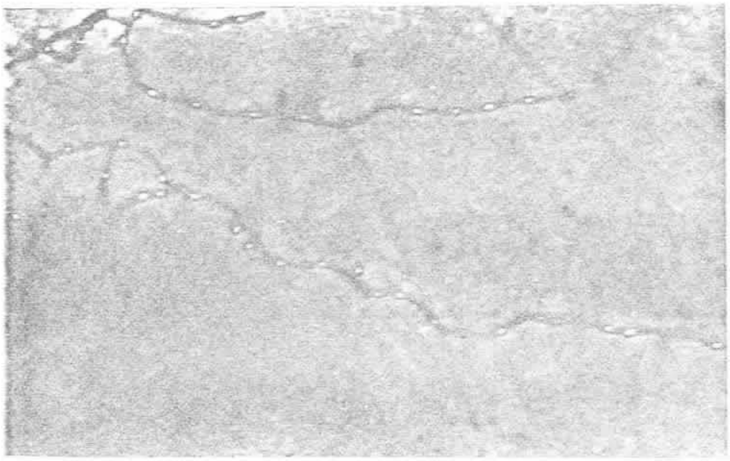

Fig. 1. Substrate hyphae with sessile spores (strain D-F-A-12), after 24-days incubation at 28 °C on starch-casein agarfilm in moist-chamber. Phase-contrast illumination.

The systematic position of the genus *Promicromonospora* KRASSILNIKOV et al. 1961 is uncertain at present. *Promicromonospora* was mentioned by LUEDEMANN in Bergey's Manual of Determinative Bacteriology (1974) among the genera "*incertae sedis*", but later (1980) it was included in the approved list of bacterial names by SKERMAN et al. (1980). The two, otherwise completely identical, strains of this genus isolated and studied previously produce aerial mycelium: this is completely lacking at our isolates. According to LECHEVALIER (cit. LUEDEMANN, in Bergey's Manual, 1974), their cell wall composition is of type Group VI. Dr. BARABÁS and his colleagues (Biological Institute of the Medical Univ., Debrecen, 1981, personal communication), who studied the composition of the cell wall of our gut-isolates detected the presence of lysine, alanine, glutamine and threonine, but both meso- and LL-diamonopimelic acids were absent. This finding confirms decisively that the species in the gut of *Ch. projectus* do not belong to Micromonosporaceae (since the members of the latter have a cell wall of type II, III or IV which all contain diaminopimelic acid) but are very closely related to *Promicromonospora*. A detailed description and a more precise taxonomic identification of this interesting new type of gut-bacteria will be given in our next publication.

4. Acknowledgement

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Actinomycetes show a very characteristic distribution pattern in litter and millipede habitats. In the litter itself, streptomycetes predominate but they have only a subordinate role in the total bacterial community of the litter. Conidia of *Streptomyces* spp. tend to adhere to and accumulate on the hydrophobic surface structures of the exoskeleton of millipedes, where they can constitute more than 90 per cent of the total microbial passengers. In the digestive canal of millipedes there is an increase in both the absolute number of actinomycetes and their relative frequency of occurrence. In contrast to the litter and body surface actinomycete populations consisting mainly of diverse streptomycete species, in the gut of *Chromatoiulus projectus* Verh. only a single *Promicromonospora*-type species can multiply and attain large population densities. Although further investigations are necessary, it is clear that the gut-flora of other Diplopoda species contains similar, large homogeneous actinomycete fractions composed of *Promicromonospora*-type organisms. These are true gut-inhabitants which do not occur outside the intestinal milieu of millipedes.

Key words: Diplopoda, Promicromonospora, gut-flora, actinomycetes.